Fluorescent Probes

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Two-Photon Fluorescent Probes for Intracellular Free Zinc Ions in Living Tissue**

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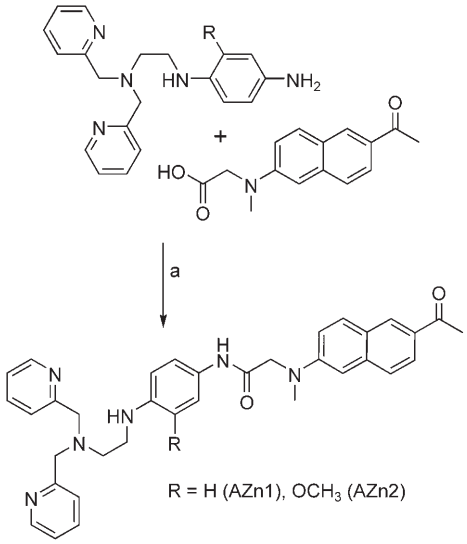
Zinc is a vital component of enzymes and proteins.[1,2] In the brain, a few millimoles of intracellular free Zn^{2+} ions are stored in the presynaptic vesicles, released with synaptic activation, and seem to modulate excitatory neurotransmission.[2] To understand the biological roles of Zn^{2+}, a variety of fluorescent probes derived from quinoline (TSQ, Zinquin, and TFLZn) and fluorescein (FluZn-3, Znpyr, ZnAF, etc.) have been developed.[3–7] However, most of them require a rather short excitation wavelength or suffer from pH sensitivity.

To visualize the biological activity deep inside living tissue (> 80 μm) without the interference of surface preparation artifacts,[8a] it is crucial to use two-photon microscopy (TPM), which utilizes two photons of lower energy for the excitation. Recently, TPM has gained much interest from biologists because it offers a number of advantages in biological imaging, including increased penetration depth, localized excitation, and prolonged observation time.[8] However, efficient two-photon (TP) probes for Zn^{2+} appear to be rare.[9] Furthermore, although a few pH-resistant sensors for Zn^{2+} have been reported, they require either microinjection for cellular applications[10] or use a significant amount of ethanol as co-solvent because of the poor water solubility.[11]

An efficient TP probe for Zn^{2+} should have sufficient water solubility to stain the cells, high selectivity for Zn^{2+} ions, significant TP cross section, pH resistance, and high photostability. In this context, we extend our earlier work[12,13] and present new TP probes for intracellular free Zn^{2+} ions (AZn1 and AZn2) derived from 2-acetyl-6-(dimethylamino)naphthalene (acedan) as the fluorophore[13] and *N*,*N*-di-(2-picolyl)ethylenediamine (DPEN) as the Zn^{2+} chelator. Acedan is a polarity-sensitive fluorophore that has been successfully employed in the design of TP fluorescent probes for the membrane[14] and metal ions,[12,13] and DPEN is a well-known receptor for Zn^{2+}.[6] Herein, we report that AZn1 and AZn2 are capable of imaging the intracellular free Zn^{2+} ions in live cells for a long period of time and in living tissue at a depth of > 80 μm without mistargeting and photobleaching problems.

The synthesis of AZn1 and AZn2 is shown in Scheme 1. The water solubilities of AZn1 and AZn2 are about 3.0 μM, which is sufficient for staining the cell (see the Supporting Information). The emission spectra showed large bathochromic shifts (> 80 nm) with the solvent polarity (E_T^N) in the order 1,4-dioxane < DMF < EtOH < H_2O, thus indicating the utility of these compounds as polarity probes (see the Supporting Information).



Scheme 1. Synthesis of AZn1 and AZn2. a) DCC (*N*,*N*′-dicyclohexylcarbodiimide), HOBt (1-hydroxybenzotriazole), CH_2Cl_2.

When small increments of Zn^{2+} were added to AZn1 and AZn2 in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer solution (30 mM, pH 7.2, $I = 0.10$), the one-photon (OP) and TP excited fluorescence intensity increased gradually without affecting the absorption spectra (Figure 1a and the Supporting Information), presumably because of the blocking of the photoinduced electron-transfer (PET) process by the complexation with Zn^{2+}. The fluorescence enhancement factors [FEF = $(F-F_{min})/F_{min}$] of AZn2 measured for OP and TP processes were 2.5-fold larger than those of AZn1 as a result of the lower fluorescence quantum yield (Φ) in the absence, and higher Φ in the presence, of excess Zn^{2+} (see the Supporting Information).

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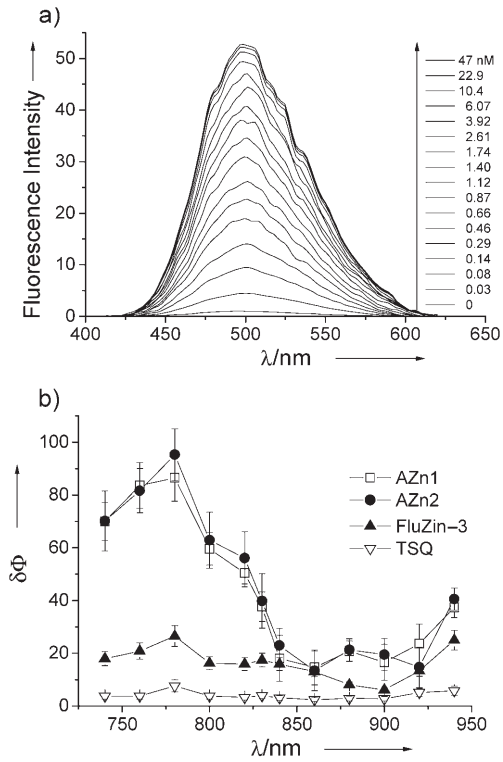

Figure 1. a) TP fluorescence spectra of 2 μM AZn2 (30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) in the presence of free Zn^{2+} (0–47 nM). The excitation wavelength is 780 nm. b) TP action spectra of AZn1 (□), AZn2 (●), FluZin-3 (▲), and TSQ (▽) in the presence of 1.8 μM free Zn^{2+}. EGTA = ethylene glycol bis(2-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid.

DFT calculations at the B3LYP/6-31G** level revealed that the highest molecular orbital energies of DPEN (R1), 2-methoxy-DPEN (R2), AZn1, and AZn2 are −4.948, −4.246, −8.762, and −8.733 eV, respectively (see the Supporting Information).[15] Hence, the PET from R2 to AZn2 might occur more efficiently on thermodynamic grounds, thereby decreasing the Φ value.[13d] On the other hand, the larger Φ for AZn2–Zn^{2+} may be attributed to the tighter binding (see below), which may reduce the vibrational relaxation pathways. Moreover, the linear Hill plots determined for Zn^{2+} binding with a slope of 1.0 indicated 1:1 complexation between the probes and Zn^{2+} (see the Supporting Information).[16] The optimized geometries of AZn1–Zn^{2+} and AZn2–Zn^{2+} complexes are trigonal bipyramidal, in which Zn^{2+} ions are coordinated by four nitrogen atoms and one water molecule (see the Supporting Information).

The dissociation constants (K_d^{OP} and K_d^{TP}) for AZn1 and AZn2 calculated from the OP and TP fluorescence titration curves[3,6a] are (1.1 ± 0.1) and (0.50 ± 0.04) nM, respectively (see the Supporting Information); the detection limits of these probes are in the subnanomolar range. The smaller K_d^{TP} value for AZn2 is consistent with the tighter binding between AZn2 and Zn^{2+} (see the Supporting Information). Both probes show high selectivity for Zn^{2+} compared with Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}, and are pH-insensitive in the biologically relevant pH range (see the Supporting Information).

The TP action spectra of the Zn^{2+} complexes with AZn1 and AZn2 in buffer solutions indicated a $\Phi\delta$ value of ca. 90 GM at 780 nm, four- to 24-fold larger than those of TSQ and FluZin-3 (Figure 1b and the Supporting Information). This finding indicates that TPM images for samples stained with AZn1 and AZn2 would be much brighter than those stained with commercial probes.

The TPM images of 293 cells labeled with both probes showed no two-photon excited fluorescence (TPEF) at 360–460 nm (Figure 2b and the Supporting Information) and appreciable TPEF at 500–620 nm (Figure 2c and the Supporting Information). For comparison, the TPM images of cells labeled with Acedan-derived TP probes for Mg^{2+} (AMg1)[13a] and Ca^{2+} (ACa1)[13b] showed TPEF in the 500–620 and 360–460 nm regions, which has been attributed to the probes associated with cytosol and membrane, respectively. Hence, AZn2 appears to be predominantly located in the cytosolic compartments, probably because of the lower molecular

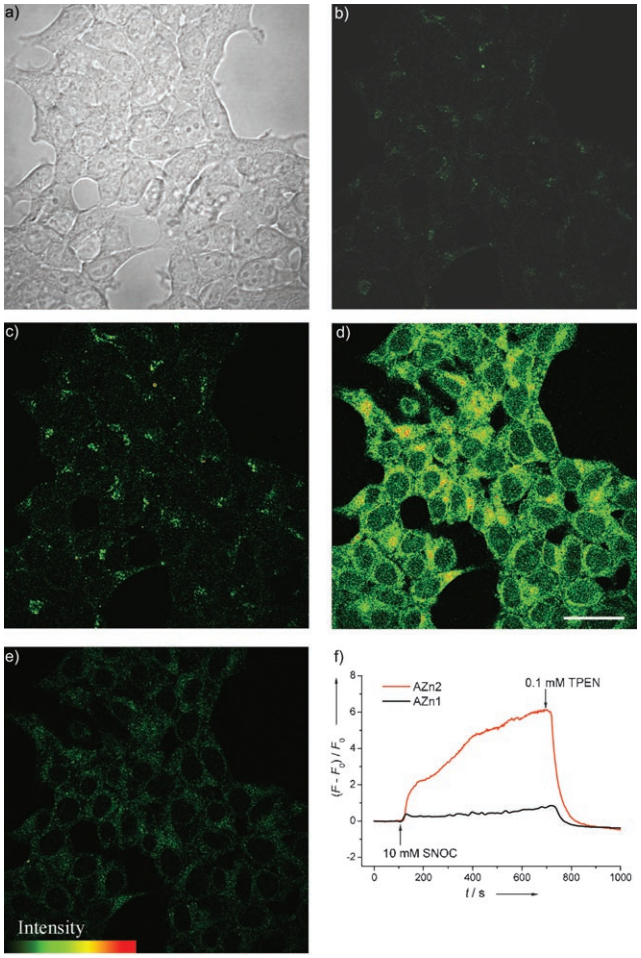

Figure 2. a) Bright-field image. b–e) TPM images of 2 μM AZn2-labeled 293 cells collected at 360–460 (b) and 500–620 nm (c–e), before (c) and after (d) addition of 10 mM SNOC to the imaging solution; scale bar: 30 μm. e) After addition of 0.1 mM TPEN to (d). f) Relative TPEF intensity of AZn1- and AZn2-labeled 293 cells collected at 500–620 nm as a function of time. The TPEF images were collected upon excitation at 780 nm with a femtosecond pulse. Cells shown are representative images from replicate experiments ($n = 5$).

weight, and thereby can detect $[Zn^{2+}]_i$ in live cells without interference from the membrane-bound probes.

Moreover, because the fluorescence intensities of AZn1 and AZn2 increase slightly at pH < 4 (see the Supporting Information), there is a possibility that the probes in the acidic vesicles might partially contribute to the TPM images. To rule out such a possibility, 293 cells and primary cortical cultures were co-stained with AZn2 and LysoTracker Red, a well-known OP fluorescent probe for acidic vesicles,[17] and the images were co-localized. However, they did not merge (see the Supporting Information). Moreover, the TPM images taken after treatment with 100 μM *N*,*N*,*N*′,*N*′-tetrakis(2-pyridyl)ethylenediamine (TPEN), a membrane-permeable Zn^{2+} chelator that can effectively remove Zn^{2+},[6,7] showed little TPEF emission (see the Supporting Information). Furthermore, the OP microscopy images taken before and after treatment with TPEN are nearly identical (see the Supporting Information). Hence, AZn2 can selectively detect $[Zn^{2+}]_i$ in neurons by TPM without interference from the probes associated with the acidic vesicles.

To demonstrate the utility of this probe in cell imaging, we monitored the TPEF of AZn2-labeled 293 cells after addition of 10 mM *S*-nitrosocysteine (SNOC), an endogenous NO donor that triggers the release of Zn^{2+}.[7b,c] The TPEF intensity increased gradually with time and then decreased abruptly upon addition of 0.1 mM TPEN, a membrane-permeable Zn^{2+} chelator that can effectively remove Zn^{2+} (Figure 2 d–f).[7b,c] A similar result was observed with AZn1 except that the response was smaller as a result of the larger K_d (Figure 2 f and the Supporting Information). Hence, these probes are clearly capable of detecting $[Zn^{2+}]_i$ in live cells for longer than 1000 s.

To further investigate the utility of this probe in deep-tissue imaging, TPM images were obtained from a part of an acute rat hippocampal slice incubated with 10 mM AZn2 for 30 min at 37 °C. Because the slice of a 14-day-old rat was too big to show with one image, several TPM images were obtained in the same plane at a depth of about 120 μm and combined. They reveal intense fluorescence in the stratum lucidum of CA3 and the hilus of dentate gyrus (Figure 3 a).[18] The image obtained at a higher magnification clearly shows that $[Zn^{2+}]_i$ is concentrated in the mossy fiber axon terminals of pyramidal neurons in the CA3 region (Figure 3 b). The negligible TPEF after addition of TPEN provides supporting evidence for this observation (Figure 3 c). Moreover, the TPM images obtained at a depth of 80–150 μm revealed the $[Zn^{2+}]_i$ distribution in the mossy fibers of dentate granule neurons near the hilus exclusively in the given plane along the *z* direction (see the Supporting Information). When 50 mM KCl, a membrane depolarizer causing the release of Zn^{2+}, was added to the imaging solution, the TPEF intensity increased and then decreased upon treatment with TPEN (Figure 3 d–f and the Supporting Information). Similar results were reported by others.[19] These findings demonstrate that AZn2 is capable of detecting intracellular free Zn^{2+} ions at a depth of 80–150 μm in living tissues by using TPM.

In conclusion, we have developed TP probes (AZn1 and AZn2) that show a 24- to 52-fold TPEF enhancement in response to Zn^{2+}, dissociation constants (K_d^{TP}) of (1.1 ± 0.1) and (0.50 ± 0.04) nM, respectively, and pH insensitivity in the biologically relevant range. They also emit four- to 24-fold stronger TPEF than TSQ and FluZin-3 upon complexation with Zn^{2+}. Better than the currently available probes, these novel probes can selectively detect intracellular free Zn^{2+} ions in live cells for longer than 1000 s and in living tissues at a depth of 80–150 mm without interference from other metal ions and the membrane-bound probes.

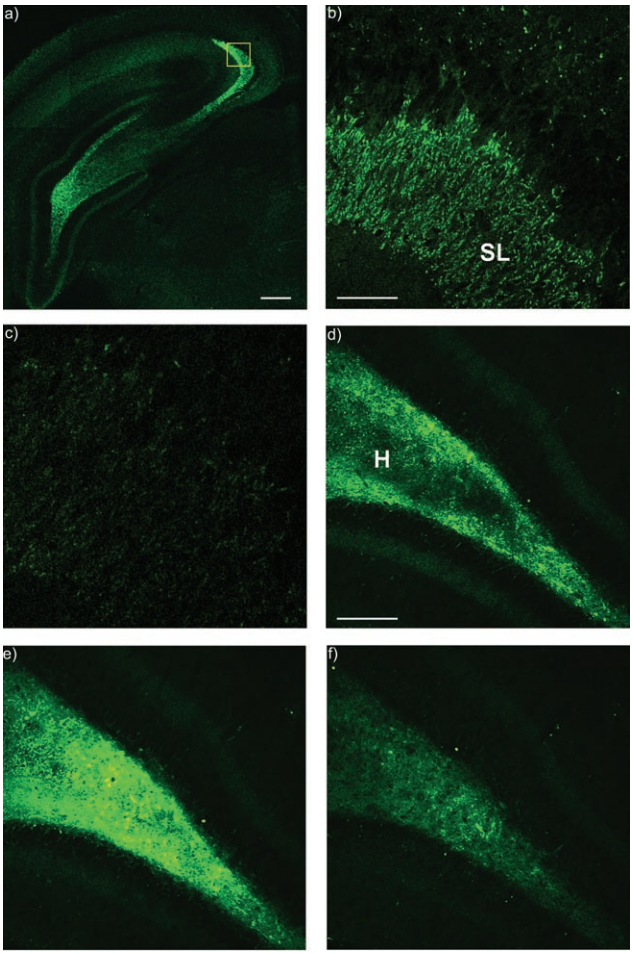

Figure 3. TPM images of a rat hippocampal slice stained with 10 μM AZn2. a) At a depth of ca. 120 μm with magnification 10×. Scale bar: 300 μm. b,c) Magnification at 100× in the stratum lucidum (SL) of CA3 regions (yellow box in (a)) at a depth of ca. 100 μm before (b) and after (c) addition of 200 μM TPEN to the imaging solution. Scale bar: 150 μm. d–f) TPM images in the hilus (H) of dentate gyrus (DG) regions at a depth of ca. 100 μm before (d) and after (e) addition of 50 mM KCl to the imaging solution. Scale bar: 300 μm. f) After addition of 200 μM TPEN to (e). The TPEF images were collected at 500–620 nm upon excitation at 780 nm with a femtosecond pulse.

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